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Tumour necrosis factor (TNF-α) in leishmaniasis

II. TNF-α-INDUCED MACROPHAGE LEISHMANICIDAL ACTIVITY IS MEDIATED BY NITRIC OXIDE FROM L-ARGININE

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SUMMARY

Peritoneal macrophages from CBA mice incubated with recombinant murine tumour necrosis factor (TNF-α) are effective in killing the protozoa parasite *Leishmania major in vitro*. The leishmanicidal activity is directly correlated with the level of nitrite (NO_2^-) in the culture supernatants. The killing of intracellular parasites can be completely inhibited by L-N^G-monomethyl arginine (L-NMMA), a specific inhibitor of the L-arginine:nitric oxide (NO) pathway. The level of NO_2^-, which is also a measurement of NO production, in the culture supernatant of TNF-α-activated macrophages can be progressively decreased to basal level with increasing concentrations of L-NMMA, but not with its D-enantiomer, D-NMMA. These data demonstrate that NO is an important effector mechanism in the TNF-α-induced macrophage killing of intracellular protozoa.

INTRODUCTION

Cell-mediated immunity is generally believed to play a causal role in the host resistance against the protozoa parasite *Leishmania* (Howard, 1985). Interferon-gamma (IFN-γ), secreted by antigen-specific $CD4^+$ Th1 cells, has been shown to be a major inducer of macrophages for the elimination of intracellular parasites (Mauel & Behin, 1982; Murray, 1982; Nacy *et al.*, 1986; Liew, 1989; Heinzel *et al.*, 1989). Recently, it has been demonstrated that tumour necrosis factor (TNF-α) can also mediate host protection against experimental murine cutaneous leishmaniasis (Titus, Sherry & Cerami, 1989; Liew *et al.*, 1990b). The mechanism for the TNF-induced host resistance is unknown.

There is now much interest in the biological role of nitric oxide (NO). It is generally accepted that NO is the endothelial-derived relaxing factor (Palmer, Ferrige & Moncada, 1987) and that it is also involved in the regulation of nervous and immune systems (Moncada, Palmer & Higgs, 1989). Activated macrophages form NO_2^- and NO_3^- from the terminal guanidino nitrogen atom of L-arginine (Hibbs *et al.*, 1987; Iyengar, Stuehr & Marlett, 1987) by a process now known to proceed via the formation of NO (Marletta *et al.*, 1988; Hibbs *et al.*, 1988). This pathway is competitively inhibited by the L-arginine analogue L-N^G-monomethyl arginine (L-NMMA) (Hibbs *et al.*, 1987; Marletta *et al.*, 1988; Palmer *et al.*, 1988). NO has been shown to be responsible for the cytotoxicity of activated macrophages against tumour cells (Hibbs *et al.*, 1987) and mediates the cytostatic effect of macrophages for the fungal pathogen *Cryptococcus neoformans* (Granger *et al.*, 1989). Moreover, macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* also involves arginine-dependent production of reactive nitrogen intermediates (James & Glaven, 1989). In the leishmanial system, the leishmanicidal activity of IFN-γ-treated macrophages can be completely inhibited by L-NMMA but not by D-NMMA (Green *et al.*, 1990; Liew *et al.*, 1990a), indicating that IFN-γ-induced killing of intracellular protozoa is mediated by NO. Furthermore, *Leishmania major* promastigotes are killed when incubated at room temperature in phosphate-buffered saline (PBS) containing NO (Liew *et al.*, 1990a). CBA mice developed exacerbated disease when L-NMMA was injected into the lesion (Liew *et al.*, 1990a). Here, we report that TNF-α-induced macrophage leishmanicidal activity is also mediated by NO.

Abbreviations: TNF-α, tumour necrosis factor-alpha; L-NMMA, L-N^G-monomethyl arginine; NO, nitric oxide.

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MATERIALS AND METHODS

Mice

CBA/T6T6 mice aged 8–10 weeks were obtained from the colonies at Wellcome Biotech, Beckenham, Kent, U.K.

Parasites

The isolation, cultivation and maintenance of the promastigote stage of the parasite *Leishmania major* (LV39) have been described in detail elsewhere (Liew, Howard & Hale, 1984).

Materials

L-N^G-monomethyl arginine (L-NMMA) and its D-enantiomer (D-NMMA) were kindly provided by Dr H. Hodson of the Department of Medicinal Chemistry, Wellcome Research

Laboratories. Recombinant murine TNF-α was kindly provided by Dr G. Adolf, Ernst Boehringer-Institut für Arzneimittel-Forschung, Vienna, Austria. The biological activity of the TNF-α preparation has been described elsewhere (Liew *et al.*, 1990b). Lipopolysaccharide (LPS) derived from *Salmonella enteritidis* was obtained from Sigma (Poole, Dorset, U.K.). [^{3}H]thymidine (26 Ci/nmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K.

Leishmanicidal assay

This has been described in detail elsewhere (Liew *et al.*, 1989). Briefly, peritoneal exudate cells were collected in culture medium [RPMI-1640 plus 10% foetal calf serum (FCS), L-glutamine, penicillin, streptomycin] from CBA mice injected i.p. 6 days previously with 3 ml of a 2% sterile, hydrolysed starch solution (BDH Chemicals, Poole, Dorset, U.K.). The cells were plated at 1×10^5 cells/0·1 ml culture medium/well in 96-well flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) and were incubated at 37° in an atmosphere of 5% CO_2 in air for 24 hr. Non-adherent cells were removed and the adherent cells washed three times with prewarmed medium. To each well were added 100 μl of medium containing 10 ng/ml of LPS and various amounts of TNF-α. In some cultures, graded doses of L-NMMA or D-NMMA were also added. Cultures were then incubated as above for a further 2-4 hr before addition into each well of 1×10^5 *L. major* promastigotes in 100 μl of culture medium containing 10 ng/ml of LPS. The mixture was cultured for a further 72 hr. At the end of 72 hr, 25 μl of supernatant were removed from each well and kept at −20° for the analysis of NO_2^- content and the cultures were washed extensively with prewarmed medium. To each well were added 100 μl of 0·01% sodium dodecyl sulphate solution in serum-free medium at 37° for 20–30 min. Schneider's medium supplemented with 30% FCS and L-glutamine was added (100 μl/well) and the cultures incubated at 28° for a further 72 hr. The cultures in three to six replicates were then pulsed with 1 μCi/well of [^{3}H]thymidine and the incorporation of radioactivity by viable parasites after 18 hr of further culturing was determined in a β-counter (β-plate, LKB 1205, Bromma, Sweden).

In some experiments, the macrophage leishmanicidal activity was determined by visual counting of intracellular parasites, as described in detail previously (Liew & Dhaliwal, 1987). Briefly, the CBA peritoneal cells obtained as above were dispensed into eight-well slides (Lab Tek tissue culture chamber slides; Miles Laboratories, Slough, Berks, U.K.) at 2×10^6 cells/0·2 ml culture medium/well. The slides were incubated for 6 hr at 37° and non-adherent cells removed by washing the wells three times with prewarmed medium. To each well was then added 200 μl of medium containing 10 ng/ml of LPS and graded doses of TNF-α. Cultures were incubated for 4 hr at 37° and 5% CO_2 followed by addition into each well of 4×10^7 promastigotes/100 μl of medium containing 10 ng/ml of LPS. At intervals of 2, 24, 48 and 72 hr the slides were removed, washed in PBS (pH 7·2) and fixed for 10 min with 1% glutaraldehyde (BDH Chemical Ltd, Poole, Dorset, U.K.). After fixing, the cells were washed three times in distilled water and stained with 0·25% Giemsa (Gurr) for 90 min. They were then destained for 10 min in PBS (pH 7·2), washed twice in distilled water and dipped in acetone, in a 1:1 mixture of acetone and xylol and finally in xylol before being mounted in mountant (Gurr). Parasite killing was estimated by determining the number of intact parasites in 200 macrophages. Some of the killed parasites were clearly visible with disintegrated membrane and cytoplasm.

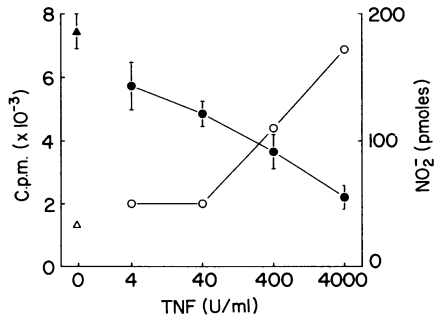

Figure 1. Induction of macrophage leishmanicidal activity and NO production by TNF-α. PEC from CBA mice were treated with graded doses of TNF as described in the Materials and Methods. The leishmanicidal activity of the macrophages was measured by the [^{3}H]thymidine incorporation method (filled symbols). Vertical bars = 1 SEM; $n = 4$. No production was measured by the content of NO_2^- (pmoles/25 μl/72 hr) in the supernatants (open symbols). Each point represents the result of a pool of four cultures. The figure is representative of three similar experiments.

Measurement of NO_2^-

NO_2^- in culture supernatants was determined by chemiluminesence as described previously (Palmer *et al.*, 1987). The levels of NO_2^- measured were indicative of NO production (Palmer *et al.*, 1987).

Statistical analysis

All experiments were performed two to three times and the results analysed by Student's *t*-test. $P < 0.05$ is regarded as statistically significant.

RESULTS

TNF-α, in the presence of LPS, induces macrophage leishmanicidal activity and the production of NO

Peritoneal exudate cells (PEC) from CBA mice were incubated with graded doses of TNF-α in the presence of 10 ng/ml of LPS and then infected with *L. major* promastigotes. The survival of the parasite in the macrophages was estimated by the visual counting of the parasite as well as by the [^{3}H]thymidine incorporation of the residual parasites. The culture supernatants were also collected for the estimation of NO_2^- production. Figure 1 shows that macrophages treated with increasing amounts of TNF-α acquired a progressive ability to kill the intracellular parasite. This leishmanicidal activity is directly correlated with the production of NO as measured by the amounts of NO_2^- in the supernatants. The reduction of the number of intracellular parasites in the TNF-treated macrophages was not due to interference of parasite uptake by the macrophages, since there was no significant difference in the number of parasites attached to or within the macrophages between TNF-α-treated or medium-treated macrophages 2–24 hr after infection (Table 1). Furthermore, destruction of the intracellular parasites in treated macrophages was clearly visible under microscopic examination at 48 and 72 hr. The leishmanicidal effect thus induced can be inhibited by a neutralizing anti-

Table 1. Effects of TNF-α and LPS on macrophage leishmanicidal activity*

TNF in culture (U/ml)	Number of *L. major*/100 macrophages†			
	2 hr	24 hr	48 hr	72 hr
4000	310±20	220±15	150±10	10±2
400	325±25	270±17	201±10	90±12
40	360±31	330±18	305±25	210±16
4	329±10	361±21	308±16	315±15
Medium	358±22	300±15	351±21	340±18

* For details, see the Materials and Methods.

† Mean of three to four cultures±1 SEM; figures underlined are significantly different ($P<0.05$) from controls (medium only).

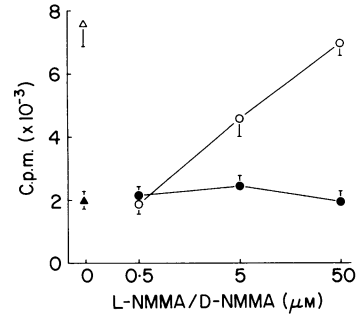

Figure 2. Inhibition of TNF-induced macrophage leishmanicidal activity by L-NMMA. PEC was treated with medium alone (△), with 4000 U/ml of TNF alone (▲), or with 4000 U/ml of TNF plus L-NMMA (○) or D-NMMA (●). Leishmanicidal activity was measured by the [^{3}H]thymidine incorporation method. Vertical bars = 1 SEM; $n=4$).

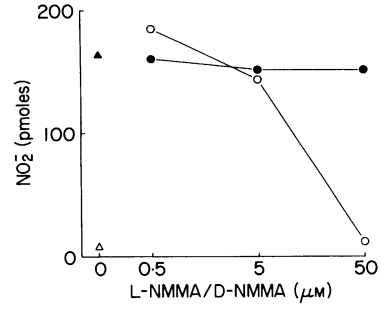

Figure 3. The 72 hr culture supernatants from the experiment described in the legend to Fig. 2 were pooled for each quadruplicate and tested for NO_2^- content. PEC treated with medium alone (△), 4000 U/ml of TNF alone (▲) or 4000 U/ml TNF plus L-NMMA (○) or D-NMMA (●).

TNF antibody (data not shown), indicating that the effect of TNF-α is not due to contaminants which may be present in the TNF-α preparation.

Effects of L-NMMA on the leishmanicidal activity

The macrophage leishmanicidal activity induced by 4000 U/ml of TNF-α was progressively inhibited with increasing doses of L-NMMA in the culture medium and was completely abrogated at 50 μM of L-NMMA (Fig. 2). In contrast, the leishmanicidal activity was not affected by equivalent concentrations of the D-enantiomer, D-NMMA.

Inhibition of NO production by L-NMMA

Figure 3 shows that the inhibition of the leishmanicidal activity by L-NMMA was accompanied by a parallel reduction of NO_2^- in the culture supernatants. NO_2^- was undetectable at a dose of 50 μM of L-NMMA. Similarly, D-NMMA had no effect on the NO_2^- production. Since the levels of NO_2^- measured were indicative of NO production (Palmer *et al.*, 1987) and that L-NMMA is a specific inhibitor of the L-arginine:NO pathway, these data demonstrate that the TNF-induced macrophage leishmanicidal activity is mediated by NO.

DISCUSSION

Earlier reports demonstrated that TNF-α plays a protective role in experimental murine cutaneous leishmaniasis *in vivo* (Titus *et al.*, 1989; Liew *et al.*, 1990). Furthermore, TNF is able to activate macrophages to kill intracellular leishmanial parasites *in vitro* (Liew *et al.*, 1990). Data presented here show that the TNF-mediated host protection against cutaneous leishmaniasis involves NO in a manner analogous to that produced by IFN-γ (Green *et al.*, 1990; Liew *et al.*, 1990a).

TNF-α has been shown to be involved in several other parasitic infections. Administration of recombinant human TNF released from intraperitoneal osmotic pumps could effectively suppress the *Plasmodium chabaudi adami* infection in CBA mice (Clark *et al.*, 1987). In experimental *Trypanosoma cruzi* infection, treatment of macrophages with recombinant TNF plus LPS resulted in a significant reduction in the number of intracellular organisms compared with controls (Wirth & Kierszenbaum, 1988). James *et al.* (1990) reported that TNF is a mediator of the macrophage cytotoxic activity against *Schistosoma mansoni*. In the latter case, the arginine-dependent production of reactive nitrogen intermediates are implicated (James & Glaven, 1989). It is thus likely that the mechanism of NO production by macrophages as a result of TNF activation in leishmaniasis applies also to these other parasitic infections.

TNF-α induces in macrophages the pathway synthesizing NO as well as the NADPH oxidase pathway producing O_2^- (Drapier, Wietzerbin & Hibbs, 1988; Ding, Nathan & Stuehr, 1988) but L-NMMA is specific for the L-arginine:NO system. It is of interest to note that the TNF-induced macrophage leishmanicidal activity can be completely inhibited by 50 μM of L-NMMA, which is not cytotoxic in itself (F. Y. Liew, unpublished data). These data indicate that NO is necessary and may be sufficient to account for the host resistance of *L. major* infection in this murine model. Whether O_2^- and H_2O_2 also play a role in this system is currently under investigation.

The precise mechanism of the microbicidal effect of NO is at present unknown. It may be similar to the mechanism by which NO from activated macrophages kills tumour cells (Hibbs *et al.*, 1990). In this system, it is postulated that exposure of Fe–S groups to NO results in iron–nitrosyl complex formation, which causes the inactivation and degradation of Fe–S prosthetic groups of aconitase and complex I and complex II of the mitochondrial transport chain (Hibbs *et al.*, 1988). Alternatively, NO may react with O_2^- to form peroxynitrite ($ONOO^-$), which decays rapidly to form the highly reactive hydroxyl radical ($HO^{\cdot}$), as suggested by Beckman *et al.* (1990). The hydroxyl radical may be the final effector in this system.

It should be noted that TNF is capable of direct lysis of a number of cell lines. For instance, the killing of L929 cells by TNF in the absence of macrophages is not inhibitable by L-NMMA (F. Y. Liew and A. Meager, unpublished data). Thus, there may be at least two different mechanisms by which TNF asserts its biological functions.

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